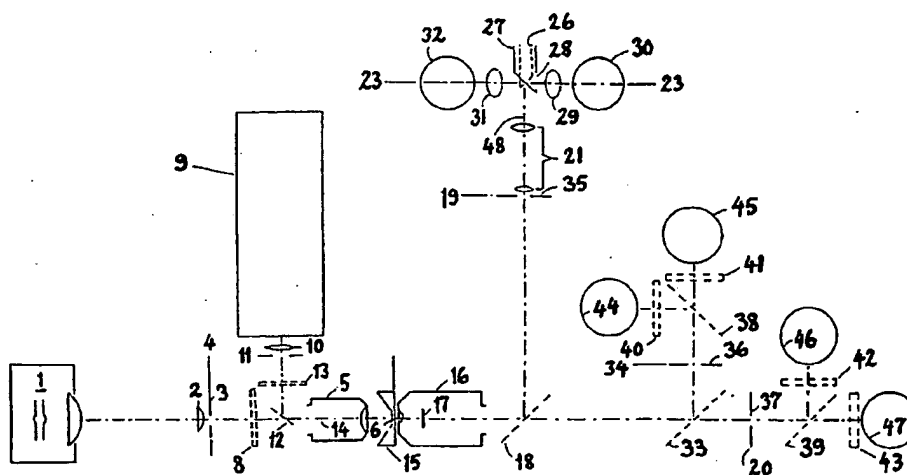


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: <b>PCT/NO94/00059</b> (22) International Filing Date: 16 March 1994 (16.03.94) (30) Priority Data: 930980 18 March 1993 (18.03.93) <b>NO</b> (71)(72) Applicant and Inventor: <b>STEEN, Harald [NO/NO];</b> Wolffsgt. 3, N-0358 Oslo (NO). (74) Agent: <b>LANGFELDT, Jens, F., C.;</b> Bryns Patentkontor A/S, P.O. Box 765, Sentrum, N-0106 Oslo (NO).</p>		<p>(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i></p>

(54) Title: AN OPTICAL ARRANGEMENT FOR FLOW CYTOMETERS



## (57) Abstract

An optical configuration for flow cytometers which allows the sample stream, which carries the cells to be measured, to be excited in two separate foci of different wavelengths. The excitation light is focussed on the sample stream by a lens (5) which has its optical axis (14) and object plane (7) coinciding with that of another lens (16) situated opposite to it and which is collecting fluorescence and light scattering from said cells and forms fluorescence images of said sample stream in different image planes (19, 20 and 34) which are separated by dichroic mirrors (18, 33). Said lens (16) contains a field stop (17) which stops direct excitation, so that only fluorescence and scattered light reach said image planes (19, 20, 34). Behind said image plane (19), which contains scattered light from said cells, is a telescope (21) which makes an image of said field stop (17) in a plane (23). In said plane (23) are two concentric mirrors (24, 25) which isolate light of small and large scattering angle, respectively, and direct this light onto separate light detectors (30, 32). By means of dichroic mirrors and band filters different spectral components of the fluorescence are separated and directed onto separate light detectors (44, 45, 46, 47). Slits (35, 36, 37) in the image planes (19, 20, 34) eliminate background light from the part of said object plane (7) which does not contain said sample flow.

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## AN OPTICAL ARRANGEMENT FOR FLOW CYTOMETERS.

The present invention relates to an optical arrangement for flow cytometers.

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A flow cytometer is an instrument for measurement of the fluorescence and light scattering of individual biological cells and other types of microscopical particles. In the flow cytometer the cells are carried by a laminar flow of water through the focus of a high intensity light source. The cells are typically stained with a fluorescent dye which binds specifically to one particular cell constituent. Each cell passing through the focus will thus emit a short pulse of fluorescence and scattered light. The intensity of the fluorescence will be proportional to the cellular content of fluorescent dye and thereby with the cellular content of the stained constituent. The intensity of the scattered light and its angular distribution is a complex function of the size, shape, structure and chemical composition of the cell. By measuring with separate detectors the light scattering at small and large scattering angles, respectively, it is thus possible to distinguish cells on the basis of size, shape, and structure.

For some purposes the cells may be stained by two or three different dyes which bind to different cellular constituents and fluoresce at different wavelengths. The corresponding spectral components of the fluorescence can be separated by dichroic mirrors and band filters and measured by separate detectors. Hence, each cell may generate several signals; typically two light scattering signals - low and large angle scattering - and two or three fluorescence signals. This technology is well known and has been published in many articles, e.g. in "Flow cytometry and sorting" (Melamed, M.R.; Lindmo, T; Mendelsohn, M.L., Eds.), Wiley-Liss, New York 1990.

35

The cellular content of the constituent(s) to be measured may be quite small, that is down to about  $1 \cdot 10^{-18}$  g/cell. The demands

on the sensitivity of the instrument are correspondingly high. In order to achieve such sensitivity the excitation light has to be concentrated into a very small and correspondingly intense focus. Furthermore, the optics which collects the fluorescence and scattered light must have the highest possible numerical aperture. It is essential also that any light from other sources than the cells, e.g. the background due to fluorescence and light scattering from optics and other components in the optical path, is as low as possible.

10

There are two major types of flow cytometers: a) Instruments employing a laser as the source of excitation light, and b) instruments using a high pressure arc lamp with xenon or mercury. The laser-based instruments have the advantage that the excitation light can be focused into a very small and correspondingly intense focus. Furthermore, the beam of excitation light is near parallel, which simplifies the distinction of light scattered to different angles. Arc lamp-based instruments have the advantage that the spectrum of the light source contains all wavelengths from UV through the visible spectrum. Hence, by means of appropriate filters the proper wavelength for excitation of any fluorescent dye can be selected, thus making this type of instruments more versatile.

25 All laser-based flow cytometers have essentially the same optical configuration, namely so that the vertical sample stream cuts through the focus of a horizontal laser beam and so that this focus is intersected at a 90 degree angle by the optical axis of the optics which collects the fluorescence and the light scattered to large angles, i.e. around  $90^\circ$ . Behind the light collecting optics fluorescence and light scattering are separated by a dichroic mirror and directed onto separate light detectors. The fluorescence may be further split into different spectral components by additional dichroic mirrors and measured by  
35 separate detectors.

The light of the focused laser beam is near parallel, that is falling within a light cone of about  $2^\circ$  or less. Hence, the light scattering at low scattering angles is measured through an other lens with its optical axis coincident with the laser beam. The  
5 laser beam is prevented from entering the lens by a field stop situated in front of the lens.

Some laser-based flow cytometers employ two lasers emitting at different wavelengths and focused to separate foci, so that the  
10 cells are excited sequentially with two different wavelengths. Thus, it becomes possible to measure two different dyes which cannot be excited by the same wavelength or which interfere in ways which are not compatible with the measurement. Such "two focus excitation" has many interesting biological applications.

15

All arc lamp-based flow cytometers employ epi-illumination, which is to say that the optics which concentrate the light in the excitation focus, also collects the fluorescence. In order to achieve the highest possible excitation intensity as well as  
20 optimal fluorescence collection efficiency, this optics should have the highest possible numerical aperture (NA). Hence, an oil immersion microscope lens, having  $NA \approx 1,3$ , is used for this purpose:

25 The large field angle of the illumination field of such a lens makes it impossible to distinguish the light scattering at small and large scattering angles by the same type of optical configuration as that used in the laser-based instruments. The Norwegian patents no. 145.176 and 156.917, as well as US-patents  
30 no. 4.408.877 and 4.915.501 disclose how light scattering can be measured in the epi-illumination type of optical configuration used in arc lamp-based instruments. By means of a central field stop close to the back focal plane of this lens, a dark field is created which allows measurement of light scattering at both  
35 small and large scattering angles through a second microscope lens situated opposite to the first and with its aperture within the dark field produced by the field stop in the first lens.

However, this configuration has certain shortcomings. Thus, it allows the light scattering at large angles to be measured only within a very small aperture, i.e.  $NA \geq 0,04$ . This small aperture limits the sensitivity of the measurement of this parameter. Furthermore, with this configuration the "large angle" range has a lower limit which does not exceed about  $20^\circ$ .

Another disadvantage of the epi-illumination configuration of current arc lamp-based flow cytometers is that it does not allow excitation in two separate foci of different wavelength, thus, limiting to some extent the range of applications of such instruments. The epi-illumination also implies that the optics which collects the fluorescence, i.e. said microscope objective, is exposed to very high intensities of excitation light. Even with microscope objectives of the very highest quality this is causing some fluorescence from the elements of the objective which adds to the background on which the cell fluorescence is detected, and thereby to a reduction of the signal to noise ratio, which is equivalent to a reduction of the sensitivity.

20

The present invention is a novel optical configuration which eliminates some of the above mentioned limitations of current designs of arc lamp-based flow cytometers. Thus, the present invention facilitates large angle light scattering measurement at considerably higher scattering angles and with a much higher numerical aperture than was feasible with the previous configuration. Hence, the light scattering sensitivity is considerably increased relative to current designs. It also produces less background light in the fluorescence light path, and allows "two focus excitation".

More specifically, the present invention provides an optical arrangement for flow cytometer, wherein intense light is focused by a microscope objective or similar lens having a numerical aperture  $NA_1$  onto a stream of cells carried by a laminar flow of water through the focal plane of said objective; and wherein another microscope lens is situated opposite to said objective,

and with its optical axis and object plane coinciding with those of said objective, and with a numerical aperture,  $NA_0$ , which is significantly larger than that of said objective; wherein said objective contains a circular central field stop in, or close to, its secondary focal plane, said field stop having a diameter corresponding to a numerical aperture,  $NA_{df}$ , which is slightly larger than  $NA_1$ , while it is much less than  $NA_0$ , so that the illumination field of said objective falls entirely within said field stop, and hence so that the image of said stream of cells created by said objective contains only fluorescence and scattered light from said stream of cells; wherein said fluorescence and scattered light from said stream of cells are separated by a dichroic mirror on basis of their different wavelength, so that said fluorescence and scattered light give rise to separate images of said stream of cells in separate image planes of said objective; and wherein a telescope, situated immediately behind said image plane creates an image of said field stop in a plane, where is situated two concentric mirrors, of different diameter, which separate light scattered from said stream of cells to different scattering angles and direct said scattered light of different scattering angles onto separate light detectors.

According to a further feature of the invention, said stream of cells coincides with said object plane of said objectives, and said stream of cells is illuminated through said objective in one or two adjacent foci of different wavelength emitted by two separate light sources.

According to another feature of the invention first and second slits may cover the image of each of said adjacent foci from said light sources in said object plane, so that fluorescence measured behind said first slit originates from only one of said foci whereas fluorescence measured behind said second slit originates only from the other of said foci.

According to yet another feature of the invention, said mirrors in said image plane of said telescope are flat, polished end planes that are cut at an angle of 45° of two concentric tubes having their common axis coinciding with the optical axis of said  
5 telescope.

The invention is now to be described with reference to a preferred, non-limitative embodiment of the invention.

10 Figure 1 shows the optical arrangement for flow cytometers, according to the invention.

Figure 2 shows more detailed a telescope formed image, according to the invention.

15

Figure 3 shows a concentric mirror embodiment, according to the invention.

The invention, shown schematically in Figures 1, 2 and 3, is a  
20 device which contains a light source 1 which, through a lens 2, illuminates an excitation slit 3, which is situated in the image plane 4 of a microscope objective or similar lens 5 which concentrates the excitation light from said light source 1 in an excitation focus 6 in the object plane 7 of said objective 5. An  
25 interference band filter 8 is situated in the light path behind said objective 5 in order to isolate the appropriate wavelength of excitation.

The device can also include a secondary light source 9 which,  
30 through a lens 10, illuminates an excitation slit 11. An image of this slit 11 is formed by said lens 5 in said image plane 7 via a dichroic mirror 12. An interference filter 13 isolates a band of excitation wavelength, preferably not overlapping that of said band filter 8. Said excitation slits 3 and 11 are  
35 situated so that their images in said object plane 7 do not overlap, but are closely adjacent on each side of the optical axis 14 of said lens 5.



The sample stream, containing cells or other microscopical particles to be measured, is conducted by the measuring chamber 15 in said object plane 7 through said optical axis 14 of said lens 5.

5

Another microscope objective 16, preferably of the oil immersion type with a numerical aperture of approximately  $NA = 1.3$ , is situated opposite said lens 5 so that the two objectives 5 and 16 have their respective optical axis 14 and respective object  
10 plane 7 coinciding.

Inside said objective 16 is a central, circular field stop 17, with its center in said optical axis 14 and in a plane which is close to the back focal plane of said objective 16. Said field  
15 stop 17 covers the central part of the aperture of said objective 16, thus stopping light falling within a solid angle corresponding to a numerical aperture,  $NA_{df}$ , which is just slightly larger than the numerical aperture,  $NA_1$ , of said lens 5. Hence, excitation light focused onto said object plane 7 by said lens  
20 5 is not transmitted by said objective 16. Consequently, the light collected by said objective 16 will contain only fluorescence and scattered light from said sample stream through said measuring chamber 15. Behind said objective 16 is situated a dichroic mirror 18 with a characteristic wavelength so that  
25 said scattered light is reflected to form an image of said sample stream in an image plane 19 of said objective 16, whereas the fluorescence is transmitted to form a corresponding image in the image plane 20 of said objective 16.

30 Behind said image plane 19 is a telescope 21 which forms an image, as shown in figure 2, of the plane containing said field stop 17 in a plane 23. Outside the dark field 22, which is the image of said field stop 17, is light scattered from cells in said sample stream. It will be understood that light falling at  
35 a given distance,  $r$ , from the center of said image in said plane 23 is emitted with scattering angles exceeding a certain limit,  $\alpha_1$  (Eq. 1) and below an upper limit,  $\alpha_2$  (Eq.2).

$$\alpha_1 \approx \arcsin[(r/r_0)(NA_{df}/n)] - \arcsin(NA_1/n) \quad \text{Eq.(1)}$$

$$\alpha_2 \approx \arcsin[(NA_0 + NA_1)/n] \quad \text{Eq.(2)}$$

5 where  $n$  is the refractive index of the sample stream, usually water, and  $r_0$  the radius of said image 22 of said field stop 17, as determined by the magnification of said telescope 21.

10 It can be seen that the lowest scattering angle which can be detected in said image plane 23, that is, at the periphery of the image 22 of the field stop 17 where  $r = r_0$ , is given by:

$$\alpha_1(\min) \approx \arcsin(NA_{df}/n) - \arcsin(NA_1/n) \quad \text{Eq.(3)}$$

15 The largest scattering angle that can be detected, i.e. at the outer periphery of the image (Fig. 2) in said image plane 23, where:

$$r = r(\max) = r_0(NA_0/NA_{df}) \quad \text{Eq.(4)}$$

20

is given by:

$$\alpha_1(\max) = \arcsin(NA_0/n) - \arcsin(NA_1/n) \quad \text{Eq.(5)}$$

25 The theory of light scattering from microscopical particles as well as experimental data on this phenomenon shows that the intensity of the scattered light falls off very rapidly with increasing scattering angle over the entire range from 0 to about  $60^\circ$ . Hence, a light scattering signal collected over a certain  
 30 range of scattering angles will be strongly dominated by scattering from angles close to the lower limit of this range. Thus, a light scattering signal collected just outside the periphery of said image 22 of said field stop 17, to a good approximation will represent low scattering angles, that is  
 35 angles just above  $\alpha_1(\min)$ ; whereas light collected close to the outer periphery contains only light from large scattering angles, that is, upwards from about  $\alpha_1(\max)$ .

A suitable value for  $NA_1$  is 0,60, whereas  $NA_{df} = 0,62$  and  $NA_0 = 1,3$ . According to Eqs. 3 and 5, these values give:  $\alpha_1(\min) = 0,97^\circ$  and  $\alpha_1(\max) = 51^\circ$ .

5 The two light scattering components representing low and large scattering angles, respectively, are directed onto separate light detectors by means of two concentric mirrors 24 and 25 (Fig. 3) formed by the plane, polished front surfaces of two cylindrical tubes which are cut at  $45^\circ$  to their axis and which are coaxial  
10 with the optical axis of said telescope 21. Said mirrors 24 and 25 face in opposite directions, as shown in Fig. 3. The inner tube 26 has an inner diameter equal to  $r_0$ , while the inner diameter of the outer tube 27 is a little less than  $r_{\max}$ . Said mirrors 24 and 25 both have their center in said image plane 23.  
15 The outer tube 27 has an opening 28 in that side which is facing said mirror 24, so that the light reflected by said mirror 24 can pass through said opening 28 and through a lens 29 to reach said detector 30. The light reflected from said mirror 25 is directed through a lens 31 onto a detector 32.

20 Between said dichroic mirror 18 and said image plane 20 is another dichroic mirror 33 which directs certain wavelengths of fluorescence, usually shorter wavelengths, to form an image from said objective 16 in the plane 34, whereas fluorescence of other  
25 wavelengths, usually longer, is transmitted to form an image in said plane 20. Thus, the device exhibits three separate image planes 19, 20 and 24 for said objective 16, wherein the same image is formed in three different regions of wavelength. In each of said image planes 19, 20, and 24 is situated a rectangular  
30 slit, the size of which can be varied so as to match the size of the image of the illuminated part of said stream of cells in said flow chamber 15 in order to eliminate light from other parts of said object plane 7 and thereby suppress background light which otherwise reduces the signal to noise ratio of the light  
35 detection and thereby the sensitivity.

Dichroic mirrors 38 and 39 and optical band filters 40, 41, 42 and 43 are situated behind said slits 36 and 37 in order to separate different spectral components of the fluorescence and direct these spectral components onto separate detectors 44, 45, 5 46 and 47.

Said dichroic mirror 18 is chosen so as to separate the scattered light, which is reflected, from the fluorescence which is transmitted because of its longer wavelength. Said dichroic 10 mirror 33 separates the fluorescence into two different spectral components, each of which is further separated by said dichroic mirrors 38 and 39. Thus, the present device can measure four different fluorescence components. This method of separating different spectral components of fluorescence is well known from 15 the literature, e.g. "Flow cytometry and sorting", Melamed et al, Wiley-Liss, New York 1990. It is trivial to increase the number of fluorescence spectral components further by the addition of more dichroic mirrors and band filters.

20 An important feature of the invention is that it facilitates so-called "two focus excitation". Light from two separate light sources 1 and 9 is passed through different band pass filters 8 and 13 which transmit two different spectral bands of excitation light. The optical axis of these two spectral bands are somewhat 25 shifted relative to each other so that said objective 5 forms two adjacent excitation foci in said object plane 7. Hence, said cells will pass sequentially through said two excitation foci. Said slits 36 and 37 are situated so that they cover the image of each of said two excitation foci. Hence, the fluorescence 30 emitted from each of said excitation foci is separated from each other and can thus be measured by separate detectors.

In the case that such "two focus excitation" is employed, one of the fluorescence detectors, for example 44 or 47 may be used to 35 measure the scattered light from cells excited in that of said excitation foci which has the largest excitation wavelength. The invention thus facilitates measurement of scattered light at two

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different wavelengths and may thereby provide further information about the cells that are being measured.

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## PATENT CLAIMS

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1.

An optical arrangement for flow cytometers, wherein intense light  
5 is focused by a microscope objective or similar lens (5) having  
a numerical aperture  $NA_1$ , onto a stream of cells carried by a  
laminar flow of water through the focal plane (7) of said  
objective (5); and wherein another microscope lens (16) is  
situated opposite to said objective (5), and with its optical  
10 axis (14) and object plane (7) coinciding with those of said  
objective (5), and with a numerical aperture,  $NA_0$ , which is  
significantly larger than that of said objective (5); wherein  
said objective (16) contains a circular central field stop (17)  
in, or close to, its secondary focal plane, said field stop (17)  
15 having a diameter corresponding to a numerical aperture,  $NA_{df}$ ,  
which is slightly larger than  $NA_1$ , while it is much less than  
 $NA_0$ , so that the illumination field of said objective (5) falls  
entirely within said field stop (17), and hence so that the image  
of said stream of cells created by said objective (16) contains  
20 only fluorescence and scattered light from said stream of cells;  
wherein said fluorescence and scattered light from said stream  
of cells are separated by a dichroic mirror (18) on basis of  
their different wavelength, so that said fluorescence and  
scattered light give rise to separate images of said stream of  
25 cells in separate image planes (19,20) of said objective (16);  
and wherein a telescope (21), situated immediately behind said  
image plane (19) creates an image of said field stop (17) in a  
plane (23), where is situated two concentric mirrors (24,25), of  
different diameter, which separate light scattered from said  
30 stream of cells to different scattering angles and direct said  
scattered light of different scattering angles onto separate  
light detectors (30,32).

2.

35 An arrangement according to claim 1, wherein said stream of cells  
coincides with said object plane (7) of said objectives (5) and  
(16), and wherein said stream of cells is illuminated through

said objective (5) in one or two adjacent foci of different wavelength emitted by two separate light sources (1,9).

3.

5 An arrangement according to claim 1, wherein first and second slits (36,37) may cover the image of each of said adjacent foci from said light sources (1,9) in said object plane (7), so that fluorescence measured behind said first slit (36) originates from only one of said foci whereas fluorescence measured behind said  
10 second slit (37) originates only from the other of said foci.

4.

An arrangement according to claim 1, wherein said mirrors (24,25) in said image plane (23) of said telescope (21) are flat,  
15 polished end planes that are cut at an angle of  $45^{\circ}$  of two concentric tubes (26,27) having their common axis coinciding with the optical axis (48) of said telescope (21).

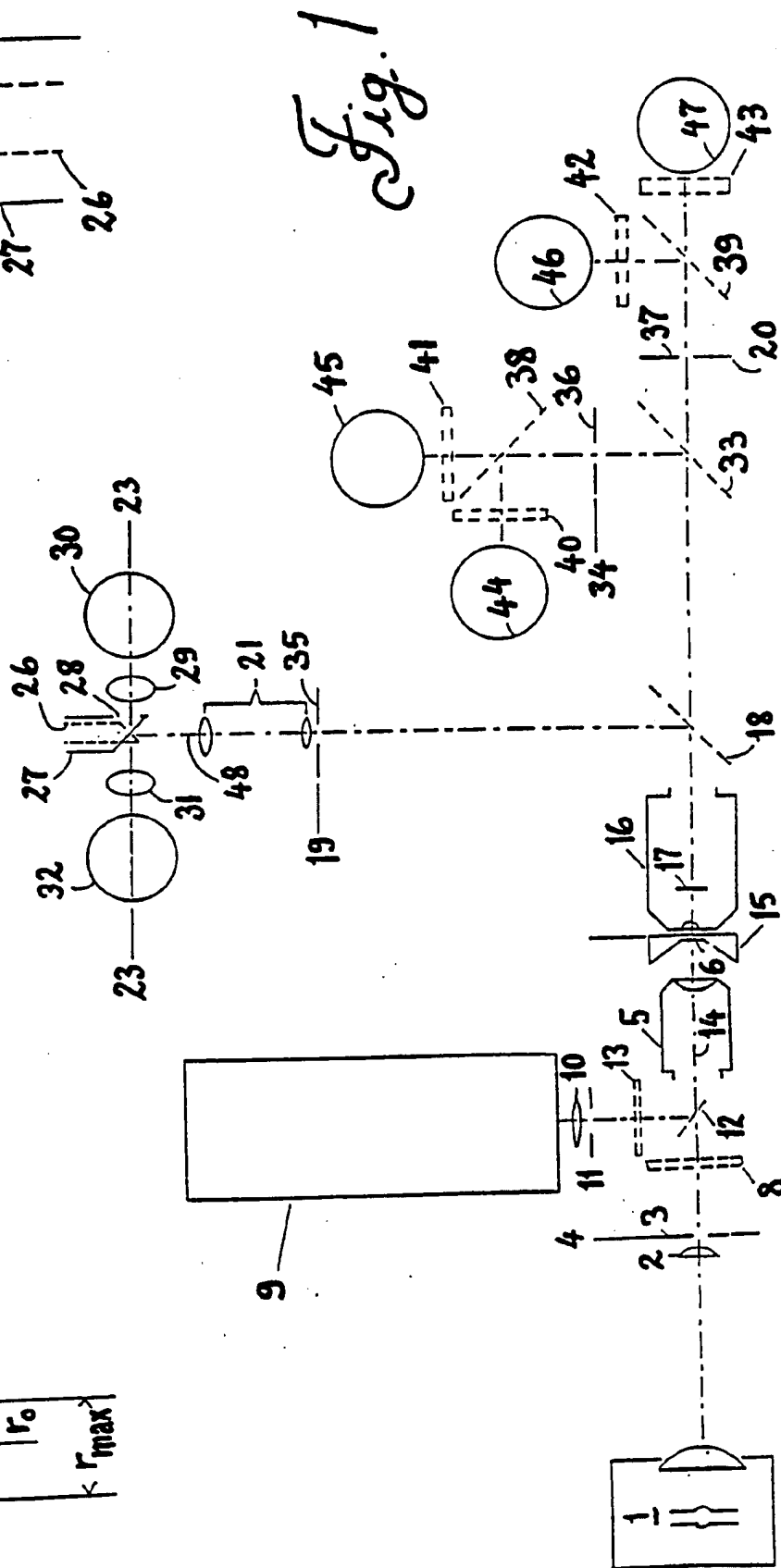
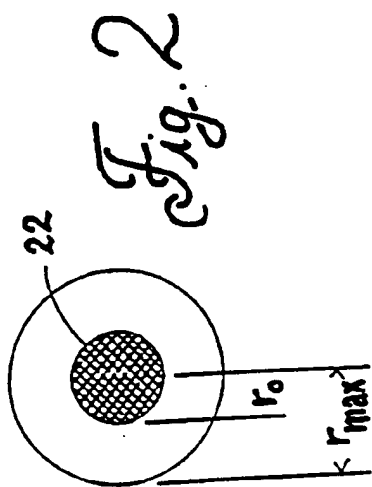
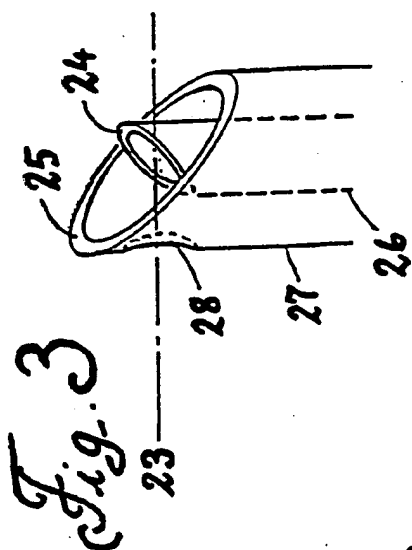
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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/NO 94/00059

## A. CLASSIFICATION OF SUBJECT MATTER

IPC5: G01N 15/14, G01N 21/53

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5185265 (HARALD B. STEEN ET AL), 9 February 1993 (09.02.93), column 2, line 35 - line 63, figure 1 --	1-4
A	WO, A1, 8700628 (STEEN, HARALD), 29 January 1987 (29.01.87), figure 1, claims 1-6, abstract --	1-3
A	US, A, 5162863 (YUJI ITO), 10 November 1992 (10.11.92), figure 1 -- -----	1

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 ☒ See patent family annex.

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Information on patent family members

28/05/94

International application No.

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